REMARKS

Claims 9-11 and 13-17 are pending. Claims 9, 11, 13, 15 and 16 have been amended. Claim 12 has been canceled. Support for new claim 17 may be found in the specification as originally filed, for example, page 6, lines 2-6.

I. The Rejection under 35 U.S.C. 112

Claims 9-14 and 16 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite.

Applicants wish to thank the Examiner for the helpful and courteous interview conducted on April 15, 2008. The "Interview Summary" dated April 22, 2008 accurately memorializes the general discussion.

II. The Rejection under 35 U.S.C. 112

Claims 9-14 and 16 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite.

In claim 9 the Examiner alleges that the phrase "in a gradient that varies in the biodegradable polymeric material" is indefinite.

Applicants respectfully submit that Applicants have clearly defined the meaning of the composition gradient of calcium phosphate. See, for example, page 6, lines 1-6 of Applicants' specification. However, for clarity, claim 9 has been amended to indicate that the gradient is a composition gradient of calcium phosphate. Applicants also respectfully submit that the term "gradient" is clear and definite in the art. See also the explanation of the term on pages 6 to 8 and in the Examples of Applicants' specification. In general, the "gradient" means that an amount of calcium phosphate varies in a biodegradable material. In certain cases, the gradient

can be checked through "concentration" of calcium phosphate (components of calcium phosphate). For example, the gradient is checked using elementary analysis which measures concentrations of calcium ion and phosphate ion. Applicants also enclose an article by an inventor which may be helpful to the Examiner in understanding the meaning of the term "gradient".

Claims 11, 13 and 15 have been amended as suggested by the Examiner. Claim 12 is cancelled. Claim 16 is amended to add a period.

For the above reasons, it is respectfully submitted that Applicants' claims are clear and definite and it is requested that the rejection under 35 U.S.C. §112 be reconsidered and withdrawn.

III. The Rejection Based on Mattern et al or Yannas et al in view of Sherwood et al

Claims 9-14 and 16 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Mattern et al or Yannas et al in view of Sherwood et al.

Applicants respectfully submit that the present invention is not anticipated by or obvious over the disclosures of Mattern et al or Yannas et al in view of Sherwood et al and request that the Examiner reconsider and withdraw these rejections in view of the following remarks.

1. Sherwood et al does not describe any biodegradable polymeric material such as glycosaminoglycan, collagen, or a composite of glycosaminoglycan and collagen

Sherwood et al describes a composite comprising tricalcium phosphate in a synthetic polymer such as PLA, PLGA, and PCL (see column 8, 5th paragraph). However, Sherwood et al does not teach or suggest or provide any reason to use a biodegradable polymeric material such as glycosaminoglycan, collagen, and a composite of glycosaminoglycan and collagen containing

calcium phosphate in a gradient so that the amount of calcium phosphate varies in the polymeric material with almost continuous (constant) gradient.

2. Tricalcium phosphate is different from normal calcium phosphate

Tricalcium phosphate used in Sherwood et al (see the Examples) is not the same as normal calcium phosphate according to the present invention. Tricalcium phosphate is a compound with formula $Ca_3(PO_4)_2$. It is also known as calcium orthophosphate, tertiary calcium phosphate, tribasic calcium phosphate, or "bone ash" and made by sintering calcium phosphate.

3. The method described in Sherwood et al can not be applied to the biodegradable material

The method described in Sherwood et al, the "3DP process", achieves the gradient of tricalcium phosphate in a device using a dispenser (dispensing module, see Fig. 1). In this method, the polymer component of the device is dissolved in an organic solvent such as chloroform, acetone, and ethanol (see column 9 line 40). However, biodegradable polymeric material such as glycosaminoaglycan and collagen cannot solve in an organic solvent. The method described in Sherwood et al cannot be applied to the biodegradable polymeric material such as glycosaminoglycan and collagen used in the present invention.

4. A continuous gradient can not be achieved by the method described in Sherwood et al

The method of the present invention can achieve almost continuous gradient of calcium phosphate in the biological material (see Example 1, Figures 3 and 4). Such a gradient can not be achieved in the method described in Sherwood et al in which tricalcium phosphate is mixed

with the polymer component at the beginning of the process.

5. Sherwood et al does not teach or provide any reason to use a gradient of calcium phosphate to achieve the effects of the present invention

Sherwood et al does not teach or suggest or provide any reason to use a gradient of calcium phosphate to achieve the unexpectedly superior effects of the present invention, *i.e.*, a biodegradable polymeric material in a composition gradient of calcium phosphate can effectively regenerate a hard/soft tissue interface.

6. Yannas et al only describe well known porous materials

Yannas et al describes porous materials such as collagen and glycosaminoglycan (GAG). Yannas et al does not teach or suggest or provide any reason to use inorganic salts such as calcium phosphate.

In the Office Action, the Examiner notes that the present claims require no method of forming the gradient that would result in a gradient different than would be obtained when soaking the scaffold in a calcium phosphate solution. Applicants' claim 9 has been amended to recite steps of making the claimed product. The gradient of calcium phosphate in a biodegradable polymeric material such as glycosaminoglycan, collagen, and a composite of glycosaminoglycan and collagen was achieved by the process of the present invention for the first time; *i.e.*, "by alternately soaking one side or part of the biodegradable polymeric material in a calcium ion-containing solution and the other side or part in a phosphate ion-containing solution."

Applicants respectfully submit that a person skilled in the art could not have made the

present invention, even if the 3DP method described in Sherwood et al were to be applied to the cross-linked collagen and glycosaminoglycan described in Mattern et al and Yannas et al.

For the above reasons, it is respectfully submitted that the subject matter of claims 9-14 and 16 is neither taught by nor made obvious from the disclosures of Mattern et al or Yannas et al in view of Sherwood et al and it is requested that the rejection under 35 U.S.C. §103(a) be reconsidered and withdrawn.

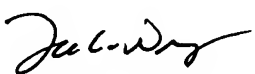
IV. Conclusion

In view of the above, Applicants respectfully submit that their claimed invention is allowable and ask that the rejection under 35 U.S.C. §112 and the rejection under 35 U.S.C. §103 be reconsidered and withdrawn. Applicants respectfully submit that this case is in condition for allowance and allowance is respectfully solicited.

If any points remain at issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the local exchange number listed below.

If this paper is not timely filed, Applicants respectfully petition for an appropriate extension of time. The fees for such an extension or any other fees that may be due with respect to this paper may be charged to Deposit Account No. 50-2866.

Respectfully submitted,
WESTERMAN, HATTORI, DANIELS & ADRIAN, LLP



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Preparation and characterization of osteochondral scaffold

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Abstract

Calcium phosphate was gradiently formed into cartilage-like matrices containing type II collagen using modified alternate soaking process. Characterization of calcium phosphate formed in the type II collagen matrices was performed using X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), thermogravimetric and differential thermal analysis (TG-DTA), and scanning electron microscopy (SEM). The results from XRD and FT-IR analysis indicated that calcium phosphate formed in the matrix was hydroxyapatite (HAp), whose phosphate ions were partially replaced by carbonate ions. TG-DTA analysis showed that HAp content increased with increasing immersion cycle in calcium and phosphate solutions. SEM image showed that a calcium phosphate layer was deposited on one side of type II collagen gels. The type II collagen gel-HAp composite with gradient calcium phosphate crystals should prove useful in regenerating the bone-cartilage interface.

Keywords: Osteochondral scaffold; Tissue engineering; Bone; Cartilage; Alternate soaking process

1. Introduction

Recently, much attention has been paid to the regeneration of orthopedic tissue such as cartilage and bone. Cartilage tissue has a very low capacity to regenerate once damaged, therefore, naturally derived polymer [1], synthetic biodegradable polymer [2,3] and hybrid materials [4] have been widely investigated as scaffolds for cartilage regeneration. Scaffolds containing calcium phosphate, such as tricalcium phosphate [5], porous hydroxyapatite (HAp) [6], and trace elements containing calcium phosphate [7] and collagen-apatite composites [8], have likewise been studied and used in bone regeneration.

Once cartilage tissue is regenerated using tissue engineering in vitro and in vivo, the engineered cartilage must be anchored at the host cartilage defect. In order to immobilize the engineered cartilage to the host tissue, various methods such as bi-compartment chambers [9] and selective differentiation technique of bone marrow stromal cells [10,11] have been employed to form cell-containing bifunctional matrices consisting of cartilage and bone (HAp) components. The resulting osteochondral tissue using above technique will show excellent adhesiveness to subchondral bone, however, the technique is complex and it takes much time to form HAp in cartilage-like matrices using cells.

HAp is formed relatively quickly on/in a three-dimensional structured organic hydrogel matrices [12–14] and polymer-grafted films [15] at room temperature and pressure in vitro using alternate soaking process. Three-dimensional hydrogel-HAp composites are easily prepared in this way.

In the present study, we developed a novel bifunctional scaffold with gradient calcium phosphate using alternate soaking process with little modification to form calcium phosphate in a cartilage-like matrix of type II collagen gels.

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2. Experimental

2.1. Materials

Type II collagen from the bovine nasal septum was donated by Nitta Gelatin (Osaka, Japan). Poly(ethylene glycol)-based 4-armed-star polymer (pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate) (4S-PEG) with branched poly(ethylene glycol) chains (n=56) was provided by NOF Corporation (Tokyo, Japan). NaH_2PO_4 and $CaCl_2$ were purchased from Wako Pure Chemical Industries, (Osaka, Japan). Tris(hydroxy methyl) aminomethane (Tris) was purchased from SIGMA CHEMICAL, MO, USA. All other chemicals were used without further purification.

2.2. Preparation of type II collagen gels

Type II collagen gels were prepared as described elsewhere [16]. Briefly, 100 μL of 4S-PEG in 0.1 M phosphate buffer saline (PBS) (pH 7.4) was added to 400 μL of type II collagen PBS solution (1.25 wt.%) at 4 °C and the final collagen concentration fixed at 1%. The mixture was then stirred and placed in molds having a silicone rubber gap 1 mm thick and 1 cm in diameter between a polyethylene plate and a silicone sheet. The cross-linking reaction was continued for 1 h at 25 °C to form calcium phosphate.

2.3. Calcium phosphate formation on/in type II collagen gels

Calcium phosphate was formed on/in type II collagen gel matrices using alternate soaking process [12–15] with minor modification. Type II collagen gels had 1 side masked with a silicone sheet in experiments. Briefly, type II collagen gels were immersed in 500 mL of $CaCl_2$ (20 mM)/0.1 M-Tris-HCl (pH 7.4) (Ca solution) at 37 °C for 5 min, then gels were removed from the Ca solution and rinsed with excess water at 37 °C. Gels were then soaked in 500 mL of Na_2HPO_4 (12 mM)/0.1 M-Tris-HCl (pH 7.4) (P solution) at 37 °C for 5 min and washed with excess water at 37 °C, forming gradient calcium phosphate in type II collagen gels.

2.4. Calcium phosphate on/in type II collagen gels

Calcium phosphate in type II collagen gels was measured in X-ray diffraction (XRD) using a diffractometer (PW1700, Philips, USA) with CuKα radiation generated at 40 kV and 50 mA at scanning angles 2θ from 5° to 60° in scanning at 2°/min. Fourier transformed infrared (FT-IR) spectra (Spectrum 2000, Perkin-Elmer, USA) of freeze-dried type II collagen gels was measured after alternate soaking in a nitrogen atmosphere using KBr pellets. For thermogravimetric and differential thermal analysis (TG-DTA; Tg8120, Rigaku, Japan), freeze-tried type II collagen gels were placed in platinum pans after different cycles. Al_2O_3 powder in another pan was used as a reference. Inorganic content in type II collagen gels was determined in an atmosphere from 25 to 800 °C at 20 °C/min heating. Scanning electron microscopy (SEM) (JSM 5600LV, JEOL, Japan) was conducted after freeze-tried gels were coated with Pt/Pd using an ion coater (ESC-101, ELINOX, Tokyo, Japan) at a 10 kV acceleration voltage.

3. Results and discussion

Fig. 1 shows a macroscopic view of type II collagen gels before and after modified alternate soaking process. Gel

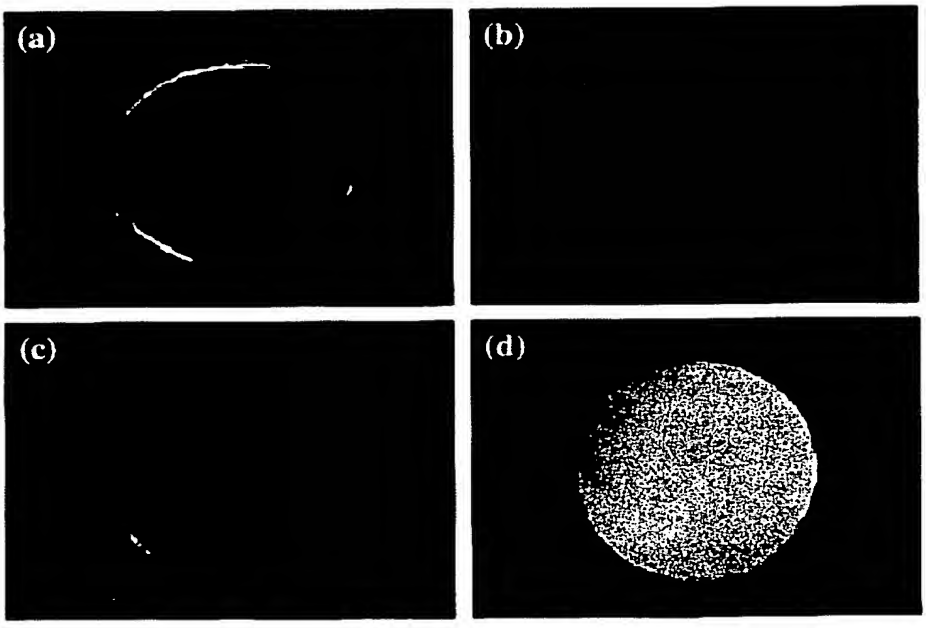

Fig. 1. Macroscopic views of type II collagen gels after modified alternate soaking process: (a) before soaking; (b) 5 cycles; (c) 10 cycles; (d) 30 cycles.

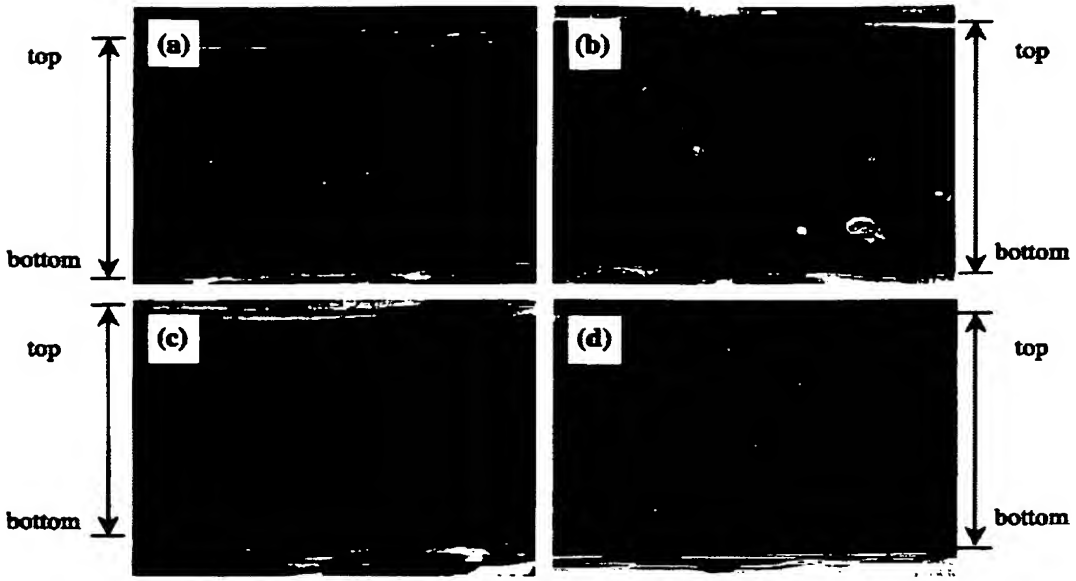

Fig. 2. Cross-sections of type II collagen gels after modified alternate soaking process: (a) before soaking, (b) 5 cycles, (c) 10 cycles, (d) 30 cycles.

water content was 99%. Type II collagen became opaque with increasing immersion time.

Fig. 2 shows cross-sections of calcified type II collagen gels after different immersion cycles. No white crystal was observed after five cycles, however, the top of type II collagen gels became opaque with increasing soaking repetition time. After 30 cycles, white crystal formed gradiently from the top to the middle of gels. Calcium phosphate was formed in a previous study on/in poly(vinyl alcohol) (PVA) gel matrices [12–14]. Similar to PVA gels, calcium phosphate formed on/in type II collagen gels. Calcium phosphate is assumed to be embedded in type II collagen gels.

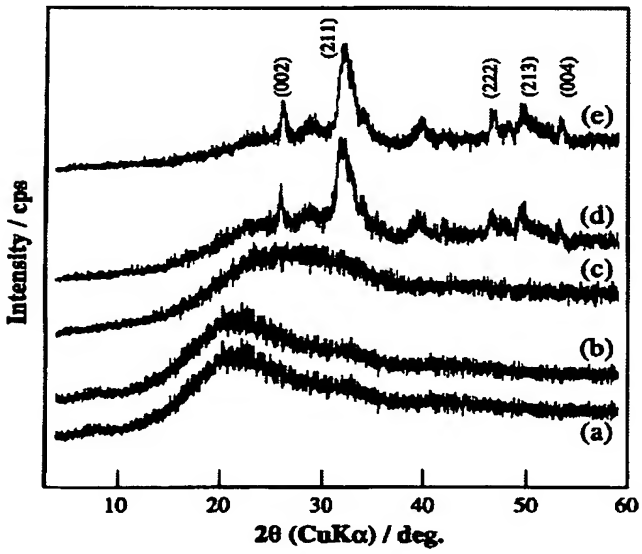

Fig. 3. X-ray diffraction patterns of type II collagen gels after different repetition cycles: (a) before soaking; (b) 1 cycle; (c) 5 cycles; (d) 10 cycles; (e) 30 cycles.

Type II collagen gels were freeze-dried for the characterization of calcium phosphate formed on/in gels. Fig. 3 shows XRD patterns of type II collagen gels after different immersion cycles. Broad halo peaks observed at 20° were attributed to the organic component type II collagen. No crystalline phase other than HAp was detected except for halo peaks. FTIR data indicated that HAP in the matrix was bone-like apatite whose phosphate ions were partially replaced by carbonate ions (Fig. 4). HAp content forming in type II collagen gels, determined from TG-DTA (Fig. 5), depended on the cycle number in gels, increasing linearly with the number of cycles up to 10 cycles. Then, the rate of HAp formation diminished at cycle numbers from 10 to 30 cycles. After 30 cycles, the inorganic content of gels was 13%. Calcium and phosphate ions diffusing into type II collagen gels is disadvantageous after an increased number of cycles because crystals in gels suppress calcium and phosphate ion permeability. The decrease in HAp formation from 10 to 30 cycles was due

Fig. 4. FT-IR spectrum of type II collagen gels after 30 cycles.

to the decreased supplementation of calcium and phosphate into gels. HAp formation on/in PVA gels was reported to increase with increasing gel water content [13] because the higher the water content of a gel, the greater the diffusion of calcium and phosphate ions. We conducted the same experiments to determine the effect of gel water content on HAp formation (data not shown). In contrast to the case of PVA gels, HAp formation increased with decreasing type II collagen gel water content. We thus expect that mechanisms behind HAp formation differ for PVA and type II collagen gels. Carboxyl groups rather than nonionic amide groups on the polymer-grafted surface appear favorable to HAp formation due to the promotion of calcium ions [15]. However, type II collagen contains carboxyl groups derived from aspartic and glutamic acids, so HAp formation on/in type II collagen gels increased with low water content due to accelerated calcium adsorption in carboxyl groups of amino acids.

Fig. 6 shows SEM images of freeze-dried type II collagen gels after modified alternate soaking process (30 cycles). The non-reaction side of type II collagen gels was porous (Fig. 6a) and free of calcium phosphate deposition. Lyophilization generally yields scaffolds having an interconnected porous structure [17], so type II collagen matrices prepared in our study will also have interconnected pores, which facilitate both cellular in-growth and nutrient diffusion from the culture medium into matrices. Pores in composite matrices ranged from 100 to 200 μm in size. Since pore size affects tissue healing response, the parameters affecting pore size in matrices must be clarified by changing the initial concentration of type II collagen, cross-linking density, and lyophilization conditions. HAp layers were observed on the opposite side of type II collagen gels as shown in (Fig. 6b), so HAp entangled in type II collagen gel matrices is expected to interact with collagen molecules. We found previously that a dense HAp layer formed on poly(acrylic acid) grafted poly(ethylene) films at densities exceeding 30 μg/cm^2 [15], so these surfaces were expected to have a hydrogel-like structure with a high affinity for calcium ions. Similar to the poly(acrylic acid) grafted surface, calcium adsorption was expected to occur on/in type II collagen gels.

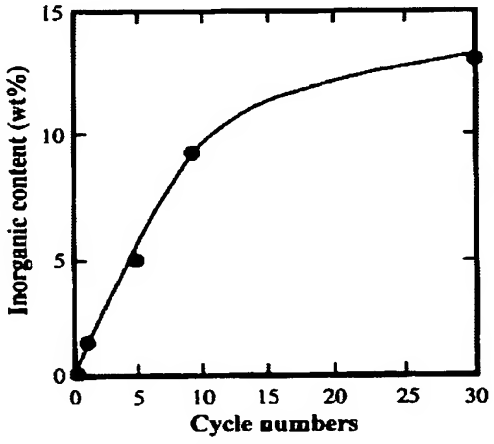

Fig. 5. Dependence of cycle numbers on HAp content in type II collagen gels.

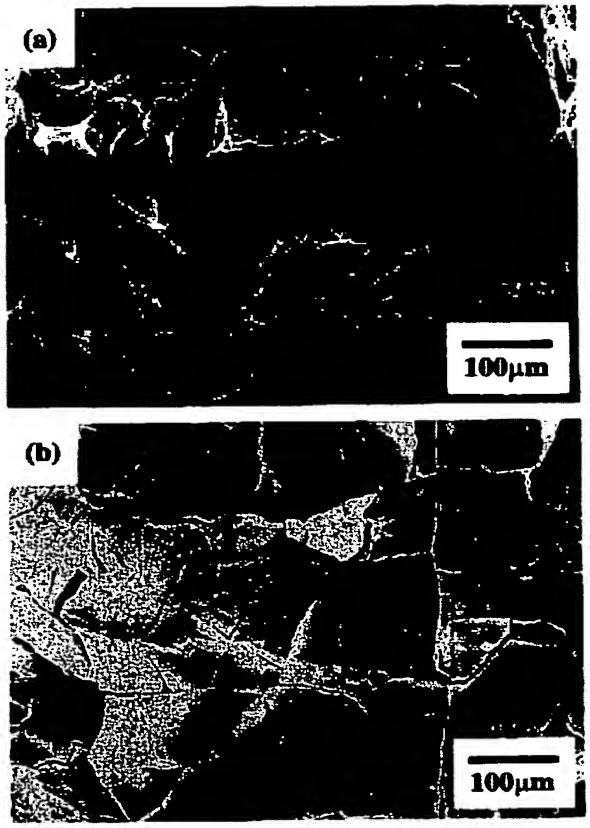

Fig. 6. SEM images of freeze-dried type II collagen gels after 30 cycles: (a) nonreaction side; (b) reaction side; (c) reaction side (magnified).

4. Conclusion

Low-crystalline calcium phosphate was gradiently formed in type II collagen gel matrices after modified alternate soaking process. The deposited calcium phosphate was HAp whose phosphate ions were partially replaced by carbonate ions. TG-DTA analysis showed that HAp content in matrices increased with increasing immersion cycles. SEM images showed that the HAp layer formed on the one side of type II collagen gels. Type II collagen gel matrices with gradient calcium phosphate crystals should prove useful in the regeneration of the bone-cartilage interface.

Acknowledgments

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References

[1] Y. Uchio, M. Ochi, M. Matsusaki, H. Kurioka, K. Katsube, J. Biomed. Mater. Res. 50 (2000) 138.
[2] K.J. Burg, W.D. Holder, C.R. Culberson, R.J. Beiler, K.G. Greene, A.B. Loebsack, W.D. Roland, P. Eiselt, D.J. Mooney, C.R. Halberstadt, J. Biomed. Mater. Res. 51 (2000) 642.
[3] Z. Gugala, S. Gogolewski, J. Biomed. Mater. Res. 49 (2000) 183.
[4] G. Chen, T. Ushida, T. Tateishi, Adv. Mater. 12 (2000) 455.
[5] Y.C. Wang, T. Uemura, R. Dong, H. Kojima, J. Tanaka, T. Tateishi, Tissue Eng. 9 (2003) 1205.
[6] A. Matsuda, T. Furuzono, D. Walsh, A. Kishida, J. Tanaka, J. Mater. Sci., Mater. Med. 14 (2003) 973.
[7] A. Ito, K. Ojima, H. Naito, N. Ichinose, T. Tateishi, J. Biomed. Mater. Res. 50 (2000) 178.
[8] M. Kikuchi, H.N. Matsumoto, T. Yamada, Y. Koyama, K. Takakuda, J. Tanaka, Biomaterials 25 (2004) 63.
[9] D.W. Hutmecher, Biomaterials 21 (2000) 2529.
[10] I. Martin, V.P. Shastri, R.F. Padera, J. Yang, A.J. Mackay, R. Langer, G.V. Novakovic, L.E. Freed, J. Biomed. Mater. Res. 55 (2001) 229.
[11] P. Angele, R. Kujat, M. Nerlich, J. Yoo, V. Goldberg, B. Johnstone, Tissue Eng. 5 (1999) 545.
[12] T. Taguchi, A. Kishida, M. Akashi, Chem. Lett. 8 (1998) 711.
[13] T. Taguchi, A. Kishida, M. Akashi, J. Biomater. Sci., Polym. Ed. 10 (1999) 331.
[14] T. Taguchi, A. Kishida, M. Akashi, J. Biomater. Sci., Polym. Ed. 10 (1999) 795.
[15] T. Taguchi, Y. Muraoka, H. Matsuyama, A. Kishida, M. Akashi, Biomaterials 22 (2001) 53.
[16] T. Taguchi, L. Xu, H. Kobayashi, A. Taniguchi, K. Kataoka, J. Tanaka, Biomaterials (2004) (in press).
[17] L.S. Liu, A.Y. Thompson, M.A. Heidaran, J.W. Poser, R.C. Spiro, Biomaterials 20 (1999) 1097.